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**13. ABSTRACT (Maximum 200 Words)**

During the second year of this career development award, we have continued to make progress in determining the contribution of the $\alpha 6$ integrin receptors to breast carcinoma progression. In previous work we had established that the $\alpha 6\beta 1$ receptor contributes to the growth and survival of breast carcinoma metastases. In addition, we had demonstrated that de novo expression of the integrin $\beta 4$ subunit in breast carcinoma cell lines that lack this integrin subunit increases their invasive potential. Since submitting the initial proposal, we have demonstrated that the ability of the $\alpha 6\beta 4$ integrin to promote carcinoma invasion is related to its activation of phosphoinositide 3-OH kinase (PI3K) and the small GTP-binding protein Rac. We have also identified PKC-epsilon as a critical effector for invasion and we have determined that it contributes to cell motility through the regulation of lamellae organization and function. We have begun to investigate how PI3K is activated by the $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins. We have preliminary evidence to support that $\alpha 6\beta 4$ cooperates with a cell surface protein for this activation. Additional signaling molecules that we are investigating that may play a role in $\alpha 6$-dependent functions are p130Cas and the tyrosine phosphatase SHP-2. An $\alpha 6$-specific ribozyme has been constructed.

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**Table of Contents**

## Introduction

Alterations in integrin expression and function during transformation are likely to have multiple consequences on tumor progression because of their adhesive and signaling properties (1,2). Our research has focused on the possible involvement of the α6 integrins, α6β1 and α6β4, receptors for the laminin family of matrix proteins, in breast cancer progression. This attention was triggered by the finding that high expression of the α6 subunit in women with breast cancer correlated significantly with reduced survival times (3). In addition, a recent report also revealed a correlation between expression of the β4 subunit and poor prognosis (4). In order to take full advantage of the α6 and β4 subunits as markers for predicting the prognosis of breast cancer, it is necessary to understand mechanistically how these integrins promote aggressive tumor behavior. Until this is established, the full potential of α6 for diagnosis, or as a target for therapeutic development, will not be known. In previous work we had established that the α6β1 receptor contributes to the growth and survival of breast carcinoma metastases (5). In addition, we had demonstrated that de novo expression of the integrin β4 subunit in colon and breast carcinoma cell lines that lack this integrin subunit increases their invasive potential (6,7). The aims of this career development award were designed to investigate these α6-dependent functions in more molecular detail. In this regard, since submitting the initial proposal we have demonstrated that the ability of the α6β4 integrin to promote carcinoma invasion is related to its activation of phosphoinositide 3-OH kinase (PI3K) and the small GTP-binding protein Rac (7). In addition, we have identified Protein Kinase C-epsilon as a downstream effector of PI3K that is required for promoting carcinoma motility and invasion.

## Body

### PKC-epsilon regulates lamellae organization and cell retraction (see appendix).

We used a genetic approach to investigate the contribution of PKC-ε and PKC-ζ to carcinoma cell migration. Transient expression of wild type and kinase inactive forms of these PKC isoforms in MDA-MB-435 breast carcinoma cells and Clone A colon carcinoma cells revealed that PKC-ε, but not PKC-ζ, is involved in carcinoma migration and invasion. Time-lapse videomicroscopy was employed to investigate the mechanism of PKC-ε function in cell motility. Cells that expressed wild type PKC-ε formed broad, polarized lamellae and PKC-ε was concentrated in membrane ruffles at the leading edge of these lamellae. Although cells expressing kinase inactive PKC-ε formed lamellae, these structures were significantly smaller and less polarized than those observed for the wild-type ε cells. These small lamellae also collapsed more quickly than the broad lamellae formed by cells that expressed wild-type PKC-ε. A defect in cell retraction was also evident in cells that expressed kinase-inactive PKC-ε. We concluded from these studies that PKC-ε is essential for carcinoma migration and invasion because it is required for the organization and stabilization of lamellae, and for the retraction of cell processes.

## Activation of PI3K by α6β1 and α6β4.

We demonstrated that the α6β4 integrin can promote carcinoma invasion through its ability to activate phosphoinositide 3-OH kinase (PI3K) and its downstream effector, the small GTP-binding protein Rac (7). The involvement of a PI3K-dependent signaling pathway in invasion is supported by other studies (8) and adds to previous data that have implicated PI3K in tumor promoting functions including transformation (9), cell survival (10, 11), anchorage-independent growth (12), and motility (13). Taken together, these findings support a central role for PI3K and its lipid products in carcinoma progression and highlight the need to investigate in more detail how this pathway is regulated. Although we have demonstrated that α6β4 can activate PI3K, a direct binding motif for the p85 regulatory subunit of PI3K is not present in the β4 cytoplasmic domain (14), suggesting that the activation of this lipid kinase is through intermediate signaling molecules. To identify these signaling intermediates, we sought to identify tyrosine phosphorylated proteins that associatiate with PI3K after ligation of the α6β1 or α6β4 receptors. As shown in Figure 1, ligation of α6β1 resulted in the association of PI3K with a phosphoprotein with an approximate molecular weight of 120kD. Ligation of the β4-transfected cells with α6-specific antibodies resulted in the association of PI3K with the 120kD phosphoprotein, as well as with a 170kD phosphoprotein. Ligation with β4-specific antibodies resulted in the association of PI3K with only the 170kD phosphoprotein. These results suggest that the α6β1 integrin activates PI3K through a 120kD intermediate whereas the α6β4 integrin activates PI3K through a distinct mechanism involving a 170kD intermediate.

To begin to identify these signaling intermediates, we examined the activation of several signaling molecules with known molecular weights of 120kD and 170kD. Two proteins that were potential candidates for the 120kD intermediate are Gab1 and Cbl because they are known to be involved in the activation of PI3K by other receptors (15,16). However, we determined that neither of these proteins is phosphorylated on tyrosine in response to clustering of α6β1 or α6β4. Another possible candidate that has been demonstrated to associate with PI3K is p130Cas. P130Cas is a docking protein that mediates the organization of signaling complexes and it has been implicated in promoting cell motility (17-19). As shown in Fig. 2, p130Cas is phosphorylated on tyrosine after clustering with α6-specific antibodies. We are presently investigating if p130Cas is the 120kD phosphoprotein that associates with PI3K after α6β1 ligation.

A potential candidate for the α6β4-specific 170kD protein that associates with PI3K is the docking protein IRS-1. This protein is phosphorylated on tyrosine and associates with PI3K in response to insulin (20). However, we determined that IRS-1 is not phosphorylated in response to α6β4 ligation. One interesting possibility is that the 170kD protein is a cell surface receptor that cooperates with α6β4 to promote signaling. We have obtained preliminary evidence to support this possibility. We labeled cell surface proteins with biotin prior to the ligation of the α6β4 receptor. As shown in Fig. 3, the 170kD protein that co-immunoprecipitates with PI3K after α6β4 clustering is biotinylated, indicating that this protein is present on the cell surface. We are presently working on obtaining additional evidence for cell surface expression of the 170kD

protein and we are working to determine the identity of this protein.

**Activation of SHP-2 by α6β4.**

To understand how α6β4 activates signaling pathways to promote carcinoma invasion, we have examined the β4 cytoplasmic domain for potential binding sites for signaling molecules. In this analysis, we identified two immunoreceptor tyrosine-based inhibitory motifs or "ITIM" motifs. The ITIM motifs were initially described in immune cell inhibitory co-receptors and have been shown to be binding sites for the protein phosphatases SHP-1 and SHP-2, and the SH2-containing inositol polyphosphate 5'-phosphatase (SHIP;21). Recruitment of these phosphatases to the β4 cytoplasmic domain could up- or downregulate the signaling functions of the α6β4 receptor by modifying the phosphorylation of β4 itself or other downstream signaling effectors. The marked increase in β4 tyrosine phosphorylation we observe upon α6β4 clustering in the presence of sodium vanadate supports the probability that a tyrosine phosphatase is activated by this receptor (Figure 4A and 4B).

We have investigated the activation of the SHP-2 phosphatase by the α6β4 integrin. We focused on SHP-2 because it is expressed ubiquitously, whereas SHP-1 is expressed primarily in hematopoetic cells. In addition, SHP-2 has been previously implicated in promoting cell motility (22). As shown in Figure 5, ligation of α6β4 results in the recruitment of SHP-2 to tyrosine phosphorylated proteins indicating that it is activated. This activation is stronger in response to ligation of α6β4 than ligation of other β1 integrins. We are presently investigating the possibility that SHP-2 associates directly with α6β4. In addition, we have obtained SHP-2 cDNAs that have been mutated in the catalytic site to investigate their potential importance in the α6β4-dependent promotion of invasion.

**Construction of α6-specific ribozyme.**

We previously demonstrated that the α6β1 receptor was important for breast carcinoma growth and survival using a dominant negative β4 subunit, β4-ΔCYT, to deplete α6β1 from the cell surface. To confirm these results, and to establish an additional system in which to examine these α6-dependent functions, we have constructed an α6-specific ribozyme that will inhibit α6 expression. This ribozyme has been transfected into the MDA-MB-435 cell line and we will identify subclones that lack α6β1 expression for our studies.

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**Key Research Outcomes:**

-PKC-epsilon is essential for breast carcinoma invasion and it functions in the organization and stabilization of actin-rich motility structures called lamellae. PKC-epsilon may also contribute to cell motility through its involvement in cell retraction.

-PI3K activation by a6b1 and a6b4 is through intermediate signaling molecules with approximate molecular weights of 120kD and 170kD respectively.

-Ligation of a6b1 and a6b4 results in tyrosine phosphorylation of p130Cas.

-Ligation of a6b4 activates the tyrosine phosphatase SHP-2.

-a6-specific ribozyme has been constructed and transfected into MDA-MB-435 cells.

**Reportable Outcomes:**

1. Manuscripts

Shaw, L.M. Integrin function in breast carcinoma progression. 1999. J. Mammary Gland Biol. and Neoplasia. (in press).

Shaw, L.M., V. Cenni, A. Toker, and A. Mercurio. PKC-ε regulates the dynamic behavior of actin-rich cell protrusions and is required for the migration and invasion of carcinoma cells. (J. Biol. Chem., submitted).

2. Abstracts

L.M. Shaw, A. Toker, and A.M. Mercurio. Protein Kinase C-epsilon is involved in the phosphoinositide 3-OH kinase-dependent promotion of carcinoma invasion by the α6β4 integrin. Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215 and Boston Biomedical Research Institute, Boston, MA 02114. (American Society for Cell Biology Annual Meeting)

3. Promoted to Assistant Professor of Medicine, Harvard University, May 1999

**Conclusions**

   The overall goal of this career development award is to understand the contribution of the α6 integrins to breast carcinoma progression. To date, we have made significant progress toward identifying α6β4-dependent signaling pathways that are involved in promoting breast carcinoma invasion. Activation of PI3K and the downstream effectors Rac and PKC-epsilon are essential for carcinoma invasion. We have begun to investigate how α6β4 activates PI3K and this information will increase our understanding of how these pathways could be manipulated for therapeutic intervention

in the future. In addition we have identified other signaling molecules, such as SHP-2, that are potentially important for $\alpha6\beta4$-dependent functions. Finally, we have made progress in establishing additional model systems for examining the role of $\alpha6\beta1$ in breast carcinoma growth and survival.

On a personal level, the work performed during the first half of this career development award has contributed to my promotion to Assistant Professor of Medicine at Harvard.

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**Appendices:**

1. Figures

2. Shaw, L.M., V. Cenni, A. Toker, and A. Mercurio. PKC-ε regulates the dynamic behavior of actin-rich cell protrusions and is required for the migration and invasion of carcinoma cells. (J. Biol. Chem., submitted).

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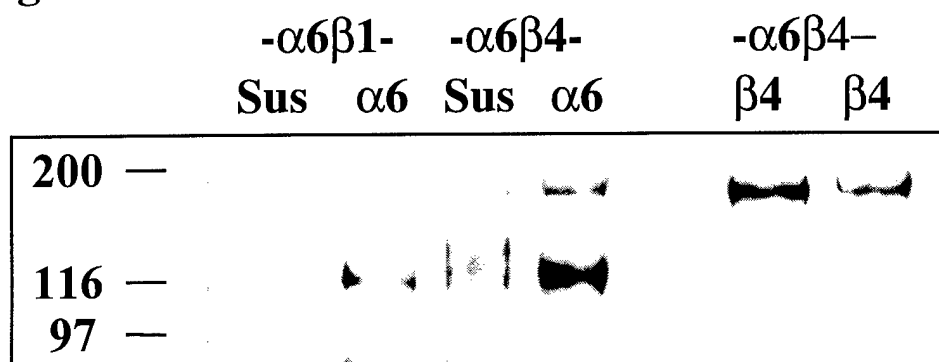
# Figure 1

|  | -α6β1- |  | -α6β4- |  | -α6β4- |  |
|---|---|---|---|---|---|---|
|  | Sus | α6 | Sus | α6 | β4 | β4 |
| 200 — |  |  |  | ▬ ◄ | ▬ | ▬ |
| 116 — | ▸ | ◄ | ▏ ▎ ▏ | ▬ |  |  |
| 97 — |  |  |  |  |  |  |

Figure 1: Tyrosine phosphorylated proteins associated with PI3K after ligation with α6β1 and α6β4. The α6β1 and α6β4 integrins activate PI3K through distinct mechanisms. Ligation of α6β1 results in the association of PI3K with a 120kD phosphoprotein whereas ligation of α6β4 results in the association of PI3K with a 170 kD phosphoprotein. Sus, cells maintained in suspension; α6, cells clustered with α6-specific antibodies; β4, cells clustered with β4-specific antibodies.

# Figure 2

|  |  | ——α6β1----- |  | -----α6β4---- |  |  |  |  |
|---|---|---|---|---|---|---|---|---|
|  | S | α6 | S | α6 | S | α6 | S | α6 |
| Phospho-<br>tyrosine<br>blot | · | ▬ | ▬ | ▬ | ▬ | ---- p130Cas |  |  |
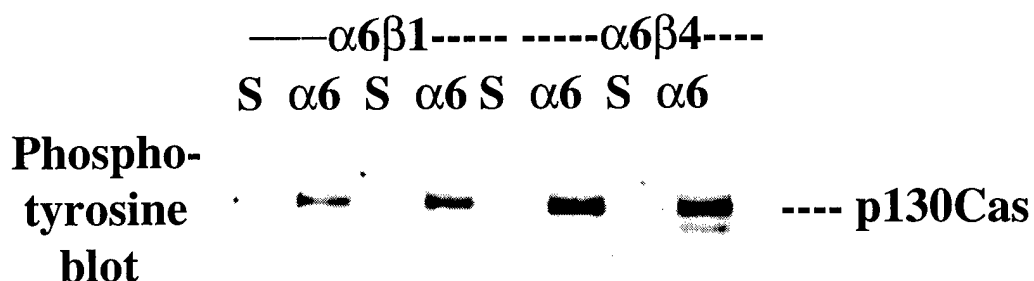
Figure 2: Tyrosine phosphorylation of p130Cas by ligation of the α6β1 and α6β4 integrins. Clustering of the α6β1 and α6β4 integrins resulted in an increase in tyrosine phosphorylation of p130Cas. A significantly greater level of tyrosine phosphorylation was observed after ligation of α6β4 than was observed after ligation of α6β1. S, cells maintained in suspension; α6, cells clustered with α6-specific antibodies.

# Figure 3

Ppt. →     α6   β4   –p85–     ——— p85 ———



Blot →     **HRP**       **Phospho-**
              **Streptavidin**     **tyrosine**
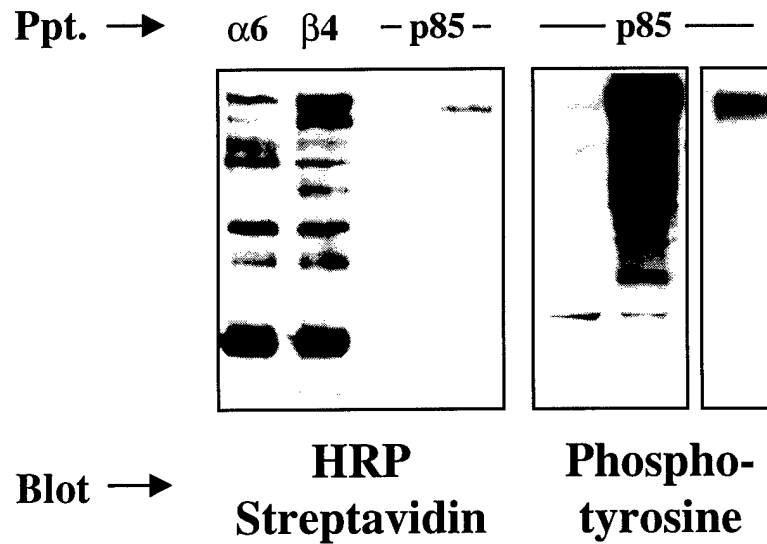
Figure 3: α6β4 cooperates with a cell surface protein to activate PI3K. The 170kD protein that associates with PI3K after α6β4 ligation is labeled by cell surface biotinylation. In the left panel, the 170 kD protein that is immunoprecipitated by PI3K-specific antibodies (p85) is detected by HRP-streptavidin indicating that it is located on the cell surface. This 170kD band co-migrates with the 170kD band that is detected by a phosphotyrosine-specific antibodies in the right panel.
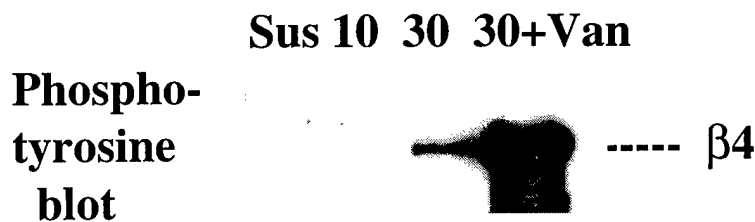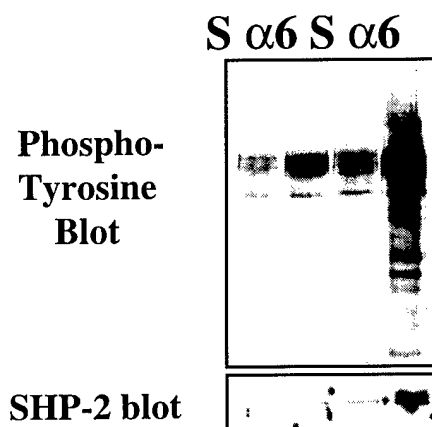
# Figure 4

**Sus 10 30 30+Van**

**Phospho-**
**tyrosine**
**blot**



----- β4

Figure 4: The β4 subunit is tyrosine phosphorylated after clustering the α6β4 receptor. The β4 subunit was immunoprecipitated from cell extracts after clustering with β4-specific antibodies for the indicated times. An increase in β4 tyrosine phosphorylation was observed with increased clustering time (10 vs. 30 minutes). Addition of sodium orthovanadate to the cells increased the level of β4 phosphorylation significantly. Sus, cells maintained in suspension; Van, sodium orthovanadate.
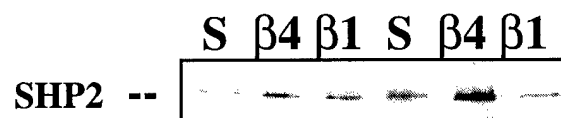
## Figure 5A

## Figure 5B

**S α6 S α6**

**Phospho-
Tyrosine
Blot**

**SHP-2 blot**

**S β4 β1 S β4 β1**

SHP2 --

Figure 4: SHP-2 is activated in response to α6β4 ligation. Ligation of the α6β4 integrin results in an increased association of SHP-2 with tyrosine phosphorylated proteins. Cells were clustered with α6-, β1-, or β4-specific antibodies and then cell extracts were immunoprecipitated with phosphotyrosine-specific antibodies. Association of SHP-2 with phosphorylated proteins indicates that it is activated. S, cells maintained in suspension; α6, cells clustered with α6-specific antibodies; β4, cells clustered with β4-specific antibodies; β1, cells clustered with β1-specific antibodies.

# PKC-ε regulates the dynamic behavior of actin-rich cell protrusions and is required for the migration and invasion of carcinoma cells

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**Running Title:** Involvement of PKC-ε in cell migration

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**Background:** Cell migration is an essential function of invasive carcinoma cells. Although many signal transduction pathways have been implicated in the control of cell migration, the mechanisms by which individual signaling molecules regulate the dynamic cytoskeletal events that underlie cell motility are relatively unknown. The protein kinase C (PKC) family is one group of signaling molecules that has been suggested to be involved in cell migration and invasion, but for which little data exist regarding either isoform specificity or their contribution to the mechanics of migration.

**Results:** In this study, we focused on PKC isoforms that are regulated by the lipid products of PI3K and used a genetic approach to investigate the contribution of PKC-ε and PKC-ζ to carcinoma cell migration. Transient expression of wild type and kinase inactive forms of these PKC isoforms in Clone A colon carcinoma cells revealed that PKC-ε, but not PKC-ζ, is involved in cell migration. The same approach revealed that PKC-ε is also essential for the *in vitro* invasion of MDA-MB-435 breast carcinoma cells. Time-lapse videomicroscopy was employed to investigate the mechanism of PKC-ε function in cell motility. Clone A cells that expressed wild type PKC-ε formed broad, polarized lamellae and PKC-ε was concentrated in membrane ruffles at the leading edge of these lamellae. Although Clone A cells expressing kinase inactive PKC-ε formed lamellae, these structures were significantly smaller and less polarized than those observed for the wild-type ε cells. These small lamellae also collapsed more quickly than the broad lamellae formed by cells that expressed wild-type PKC-ε. A defect in cell retraction was also evident in cells that expressed kinase-inactive ε.

**Conclusions:** PKC-ε is essential for carcinoma migration and invasion because it is required for the organization and stabilization of lamellae, and for the retraction of cell processes.

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## Background

An essential function of invasive carcinoma cells is migration [1]. In contrast to normal epithelial cells, which are polarized and exhibit stable cell-cell and cell-matrix interactions, invasive carcinoma cells often display a mesenchymal phenotype and their ability to migrate contributes to tumor spread [2]. For this reason, the mechanisms that underlie cell migration are critical to understanding carcinoma progression. Recent studies have highlighted the complexity of cell migration with respect to both cytoskeletal dynamics and signal transduction. At the leading edge of migrating cells, polymerization of actin filaments, organization of actin filament networks and the formation of novel adhesive contacts occur [3, 4]. In addition, translocation or actual cell movement usually involves retraction and detachment of cell adhesive contacts at the trailing edges of migrating cells [5]. Knowledge of how these dynamic cytoskeletal events are regulated by extracellular stimuli that promote the migration of carcinoma cells is needed to increase our understanding of this fundamental aspect of invasive cancer.

Many signaling molecules have been implicated in cell migration including protein kinases (i.e. MAPKs, FAK, src, PKC, ROCK; [6-10]) protein phosphatases (i.e. SHP-2, PEST; [11-13]), lipid kinases (i.e. PI3K; [14, 15]), lipid phosphatases (i.e. PTEN; [16, 17]), and GTPases (i.e. Ras, Rho, Rac, Cdc42; [18]). However, the mechanisms by which these molecules influence specific cell functions and cytoskeletal dynamics to facilitate migration are not fully understood. Recently, however, progress has been made in this direction. For example, we and others demonstrated that phosphoinositide 3-OH kinase (PI3K) is essential for carcinoma motility and invasion [15, 19]. Although the lipid products of PI3K act as second messengers that activate multiple downstream effectors including the serine/threonine kinase AKT/PKB and the small GTP-binding protein Rac, we provided evidence that Rac but not AKT/PKB contributes to migration and invasion [15]. In addition, we observed that activation of PI3K and Rac enhanced the formation of large, actin-rich protrusions termed lamellae that underlie chemotactic migration [15]. The studies on PI3K provide a paradigm for studies aimed at understanding how other signaling molecules and pathways contribute to cell migration.

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The PKC family of serine/threonine kinases represent a salient example of signaling molecules that have been implicated in cell motility but for which little, detailed mechanistic data exist [20, 21]. The PKC family consists of 11 isoforms that are classified by their mode of regulation: the conventional ($\alpha$, $\beta$I, $\beta$II, $\gamma$) are regulated by diacylglycerol (DAG) and $Ca^{++}$; the novel ($\delta$, $\epsilon$, $\eta$, $\theta$) are regulated by DAG but are independent of $Ca^{++}$,; and the atypical ($\zeta$, $\iota$, $\lambda$) are independent of both DAG and $Ca^{++}$ [22, 23]. The use of broad specificity pharmacological inhibitors that do not discriminate among the individual isoforms has precluded the identification of specific PKC isoforms that participate in cell migration. In addition, the potential mechanisms by which PKC isoforms function in cell migration need to be established.

To evaluate the contribution of specific PKC family members to cell migration rigorously, we are using a genetic approach. In this study we focused our efforts on PKC-$\epsilon$ because this isoform is activated by PI3K and PLC-$\gamma$ signaling pathways, both of which have been demonstrated to promote cell migration [24]. In addition, PKC-$\epsilon$ has been implicated in the regulation of cell spreading [25, 26]. We also assessed the involvement of PKC-$\zeta$ because the activity of this isoform is regulated by PI3K as well [22]. The results obtained implicate a critical role for PKC-$\epsilon$ in the migration and invasion of carcinoma cells because its activity contributes to the dynamic behavior of actin-rich cell protrusions.

## Results

### Involvement of PKC-$\epsilon$ in carcinoma cell migration

Clone A colon carcinoma cells were used initially to investigate the involvement of specific PKC isoforms in cell migration. These cells exhibit rapid, chemokinetic migration on laminin-1 that is characterized by the formation of fan-shaped lamellae [27]. To examine the contribution of PKC-$\epsilon$ and PKC-$\zeta$ to the migration of Clone A cells, wild-type PKC-$\epsilon$ (WT-$\epsilon$) and PKC-$\zeta$ (WT-$\zeta$), as well as kinase-inactive mutants of PKC-$\epsilon$ (KI-$\epsilon$) and PKC-$\zeta$ (KI-$\zeta$) were expressed transiently in these cells. Expression of the PKC isoforms was confirmed by immunoblotting using FLAG-specific antibodies (Fig. 1A, lower panel). In addition, *in vitro* kinase assays were

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performed to assess the relative activity of the PKC proteins in Clone A cells. As shown in Fig 1B, both the KI-ε and -ζ proteins exhibited decreased kinase activity relative to their corresponding WT-proteins.

The migration of clone A cells that expressed the various PKC proteins toward a laminin gradient was assessed using a modified Boyden chamber. As shown in Fig. 1A, expression of WT-ε increased the migration of Clone A cells by 2.5 fold. An increase in cell migration was not observed in cells that expressed WT-ζ. In the converse experiment, expression of dominant-negative KI-ε, but not KI-ζ, inhibited the migration of Clone A cells by 80% (Fig. 1A). Taken together, these results suggest that PKC-ε is essential for the migration of Clone A cells.


## Localization of PKC-ε to membrane ruffles and lamellae

To obtain additional insight into the function of PKC-ε in cell migration, we examined the morphology of Clone A cells that had adhered to laminin-1 after transfection with the WT and KI-PKC constructs, and we examined the spatial distribution of the transfected PKC proteins using indirect immunofluorescence microscopy. Cells that expressed the PKC proteins were fixed after attachment to laminin-1 for 45 minutes and the exogenous PKC isoforms were detected using a FLAG-specific mAb. Two representative cells from each transfection are shown in Fig. 2. The morphology of Clone A cells that expressed WT-ε did not differ significantly from the morphology of cells transfected with a vector alone (data not shown). These cells were polarized and displayed broad lamellae with extensive membrane ruffling (Fig. 2A,B and Fig. 3A). Interestingly, PKC-ε was concentrated in these membrane ruffles at the leading edge of lamellae (Fig. 2A,B;arrow). PKC-ε was also expressed throughout the cytoplasm and it was absent from the nucleus. The morphology of the Clone A cells that expressed WT-ζ was similar to the morphology of the cells expressing WT-ε and the WT-ζ protein was also distributed diffusely throughout the cytoplasm. Although the WT-ζ protein was observed in membrane ruffles, the concentration of this PKC isoform in these structures was much lower than that observed for the WT-ε protein. (Fig 2F; arrow).

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Clone A cells that expressed dominant negative KI-ε attached to laminin-1 to the same extent as cells that expressed WT-ε (data not shown), an observation that discounts a role for PKC-ε in regulating cell adhesion. However, cells that expressed KI-ε displayed a disorganized morphology as evidenced by their lack of a polarized leading edge and well-developed lamellae (Fig. 2C,D). Moreover, irregular membrane protrusions were observed and many long, filipodial-like extensions were also present. The KI-ε protein was distributed throughout the cytoplasm in a grainy, punctate manner and displayed some concentration at the membrane edge (Fig. 2C, arrow). Expression of the mutant PKC-ε was also observed in the filipodial-like extensions. The morphology of cells expressing KI-ζ and the localization of the KI-ζ protein were similar to that observed for the WT-ζ transfectants (Fig. 2G,H).

**PKC-ε is required for organization of lamellae and retraction of cell processes**

The localization of PKC-ε to membrane ruffles in lamellae and the lack of these structures in the cells expressing the dominant negative PKC-ε suggested that this PKC isoform functions in the formation of these actin-rich structures. To explore further the contribution of PKC-ε to lamellae function, we analyzed the movement and behavior of cells using time-lapse videomicroscopy. After attachment to laminin, Clone A cells extend filipodia, which serve as guides for the extension of lamellae [27]. Subsequently, the cell body translocates in the direction of the developed lamellae. At the trailing edge of the cell, retraction fibers are formed as the cell body moves forward and these fibers eventually detach from the matrix. This pattern of movement was observed for Clone A cells that expressed WT-ε. As shown in Fig. 3A, Clone A cells that expressed WT-ε formed organized, leading edge lamellae with active membrane ruffles and these cells migrated in the direction of lamellae formation (white arrow). As the cell moved, retraction fibers were formed at the trailing edge of the cell (Fig. 3A, black arrow).

The dynamic behavior of Clone A cells on laminin that expressed the dominant negative KI-ε protein, as assessed by videomicroscopy, revealed several important details about the function of PKC-ε. Although these cells were capable of forming lamellae (Fig. 3B;arrowhead),

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the lamellae formed were significantly smaller and not organized in a polarized manner as observed in the WT-ε transfectants. Moreover, the lamellae that formed in the KI-ε transfectants were less stable and collapsed more rapidly than the lamellae that formed in the untransfected and WT-ε transfected cells. Interestingly, cells that expressed the KI-ε construct also exhibited defects in their retraction of cell processes. When the small lamellae collapsed in these cells, thin retraction fibers were evident. Over time, many of these extensions formed, which resulted in the "filopodial-like" appearance that was observed in the fixed cells. Although filipodia were identified in the KI-ε expressing cells, most of these extensions were determined to be retraction fibers from the videomicroscopic analysis. Interestingly, small lamellae extended along these retraction fibers, similar to the formation of lamellae in the direction of filipodia as described above (small arrow; Fig. 3B;[27]). Collectively, the data obtained from time-lapse videomicroscopy indicate that PKC-ε contributes to the organization and stabilization of lamellae. In addition, PKC-ε activity is required for efficient retraction of cell processes.


**PKC-ε functions in carcinoma invasion**

The data we obtained on the involvement of PKC-ε in the migration of Clone A cells provided a rationale for assessing the importance of this PKC isoform in an *in vitro* model of carcinoma invasion. In previous work, we established that expression of the α6β4 integrin in MDA-MB-435 breast carcinoma cells stimulates their invasive potential by a mechanism that involves activation of PI3K and Rac, signaling events that culminate in the formation and stabilization of lamellae [15]. To assess the relative contribution of PKC-ε in invasion, we expressed the PKC-ε and -ζ cDNAs in the MDA-MB-435 cells that also expressed the α6β4 integrin (MDA-MB-435/β4). An additional dominant negative mutant of PKC-ε, T566A-ε, was also used. This cDNA contains a mutation at threonine 566 in the activation loop that does not permit activation of this PKC isoform. Expression of the PKC proteins after transient transfection into the MDA-MB-435/β4 cells was confirmed by immunoblotting using a FLAG-specific mAb (Fig. 4A; lower panel). In addition, *in vitro* kinase assays were performed to assess the relative

activity of the PKC constructs in the MDA-MB-435/β4 cells (Fig. 4B). As was observed with Clone A cells, the activity of the KI-ε and -ζ proteins was diminished in comparison to their respective wild type proteins. The activity of the T566A-ε mutant protein was also decreased confirming that this mutant functions as a dominant negative PKC-ε protein as well.

The ability of the MDA-MB-435/β4 cells that expressed the different PKC proteins to invade through a Matrigel-coated filter was examined. As shown in Figure 4A, expression of either the WT-ε, WT-ζ, or KI-ζ PKC proteins did not alter the invasion of MDA-MB-435/β4 cells significantly in comparison to control cells. In contrast, expression of either of the dominant negative PKC-ε mutants inhibited invasion by 50-60%. These data implicate an essential role for PKC-ε in the invasion of MDA-MB-435/β4 cells and, together with the data obtained for the Clone A cells, support a general role for PKC-ε in carcinoma migration and invasion.

Interestingly, expression of WT-ε in Clone A cells increased their migration by 2.5 fold but did not significantly increase the invasion of the MDA-MB-435/β4 cells. This discrepancy could be explained by differences in the levels of endogenous PKC-ε expression and activity. As shown in Fig. 5, a relatively low level of expression of PKC-ε was detected in Clone A cells in comparison to the level detected in the MDA-MB-435/β4 cells. More importantly, a doublet was detected by the PKC-ε antibodies in the MDA-MB-435/β4 cells and not in the Clone A cells. The more slowly migrating band of the doublet represents PKC-ε that is phosphorylated and active and only the faster migrating, less active form of the protein is present in the Clone A cells (A. Toker, unpublished data). If the levels of PKC-ε detected in the MDA-MB-435/β4 cells are optimal for function, expression of endogenous WT-ε would not increase motility further. It should be noted that the more slowly migrating, phosphorylated form of PKC-ε was detected in the WT-ε transfected Clone A cells indicating that the exogenous PKC-ε is active in these cells (Fig. 1A; lower panel).

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## Discussion

In this report, we implicate an important role for PKC-ε in the migration and invasion of carcinoma cells and define its contribution to the mechanics of migration. Our use of a genetic approach allowed a rigorous assessment of the function of specific PKC isoforms in the mobile behavior of two-well studied carcinoma cell lines, Clone A colon carcinoma cells and MDA-MB-435 breast carcinoma cells. Specifically, the data we obtained discounted the involvement of PKC-ζ in migration and invasion. However, we observed that overexpression of PKC-ε increased the rate of migration significantly, whereas expression of a dominant negative PKC-ε inhibited migration. More importantly, the use of time-lapse videomicroscopy to analyze the sequence of events involved in migration revealed that PKC-ε contributes to the organization and stabilization of actin-rich protrusions termed lamellae that are essential for cell movement. The activity of PKC-ε is also required for the efficient retraction of cell processes. Taken together, these findings substantiate the importance of PKC activity in the migration and invasion of carcinoma cells and they increase our understanding of how the actin cytoskeleton is regulated by signaling pathways that promote cell migration.

Much of the information obtained to date on actin polymerization and re-organization in response to external stimuli has focused on the Rho family of small GTP-binding proteins [18, 28]. In contrast, our knowledge of how other signaling molecules influence the organization of the actin cytoskeleton is not as well developed. Although Clone A cells that express dominant-negative PKC ε can form lamellae, these structures are smaller and less stable than the lamellae that are formed in the parental cell line. This finding suggests that the signals to initiate lamellar formation do not involve PKC-ε and that this isoform functions by regulating downstream components in this pathway. A number of regulatory and cytoskeletal proteins are required for lamellae organization including proteins that nucleate actin polymerization, promote actin filament cross-linking and promote filament disassembly [29]. Although much is known about the functions of these actin-modifying proteins, the mechanisms for how each of these proteins are regulated by the stimuli that promote lamellae formation have not been elaborated fully and need to

be investigated further. Our identification of PKC-ε as an essential regulator of lamellae organization and stabilization provides a focus for these future studies.

The ability of a kinase inactive mutant of PKC-ε to inhibit cell motility indicates that the activity of PKC-ε is essential for its function in cell migration and that downstream substrate(s) of this kinase are involved, either directly or indirectly, in regulating the morphological events that underlie cell movement. To date, none of the actin regulatory or structural proteins that are involved in lamellae formation have been reported to be phosphorylated directly by PKC-ε, although some, including ABP-280 and MARCKS, can be phosphorylated in a PKC-dependent manner [30, 31]. Unfortunately, the isoform specificity of this phosphorylation has not been determined. An alternative possibility is that PKC-ε promotes lamellae organization through an indirect mechanism. In this regard, tyrosine phosphorylation of the docking protein p130Cas is promoted by PKC-ε activity [32]. Tyrosine phosphorylation of p130Cas and its association with the adaptor protein Crk have been implicated as a "molecular switch" for promoting cell migration [33, 34]. Although the actual function of p130Cas in cell migration has not been determined, it is unlikely that it influences actin organization directly given its role as a docking protein. A more likely function for p130Cas is in the organization and localization of signaling complexes that are required for cell migration, and we suggest that such complexes may be regulated by PKC-ε.

The inability of Clone A cells that lack PKC-ε function to retract cell processes efficiently suggests that PKC-ε could be involved in regulating adhesive strength, or interestingly, in cell contraction [5]. Tension is required for a migrating cell to disrupt adhesive contacts and retract cell processes and this tension is generated by contraction of the cell body [5]. The finding that PKC-ε may be involved in cell contraction is intriguing in light of the established role for PKC-ε in smooth muscle contraction [35]. The $Ca^{++}$-independent contraction of smooth muscles is mediated by PKC-ε-dependent phosphorylation of the F-actin binding protein calponin [35]. When bound to actin filaments, calponin inhibits the actin-activated MgATPase activity of myosin and, in doing so, prevents smooth muscle contraction. Phosphorylation of calponin by PKC-ε releases this inhibition and allows contraction to occur [36]. In many non-smooth muscle cells, PKC-ε binds to

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F-actin when it is activated and this translocation from the cytosol to the actin cytoskeleton localizes the kinase to the appropriate site for the phosphorylation of calponin [37, 38]. Although calponin is expressed primarily in muscle cells, a non-smooth muscle isoform of calponin, calponin h2, has recently been identified that has a more ubiquitous expression pattern [39, 40]. This novel calponin isoform could play a role, downstream of PKC-ε, in the actin/myosin contractions that are required for cell migration.

PKC-ε is a novel PKC family member that is activated by stimuli that promote PLC-γ-dependent hydrolysis of PI-4,5-$P_2$ to produce DAG [22]. In addition, PKC-ε associates with phosphoinositide-dependent kinase-1 (PDK-1), a serine/threonine kinase that is dependent upon PI3K for function because the D3 phosphoinositide lipid products of PI3K bind to the PH domain in PDK-1 and recruit the kinase to the plasma membrane where it has access to some of its substrates [41-43]. Several of the PKC isoforms including PKC-βII, -ζ, -δ, and -ε, are phosphorylated by PDK-1 and this phosphorylation is essential for their activation ([44-46] A. Toker, unpublished data). A role for PLC-γ in promoting PKC-ε-dependent migration is supported by the fact that stimulation of chemotaxis by a number of growth factors including PDGF-β and EGF is dependent upon PLC-γ activation [47, 48]. However, to complicate matters, this growth factor-dependent chemotaxis can also require activation of PI3K [48]. Although it remains an open question at this time, it is highly likely that PLC-γ and PI3K signaling pathways cooperate to activate PKC-ε to promote cell migration. With regard to the involvement of PI3K in PKC-ε activation, we recently demonstrated that the PI3K-dependent chemoinvasion of carcinoma cells requires the small GTPase Rac, but that other PI3K effectors are also essential because Rac is not sufficient to promote invasion on its own [15]. A model could be proposed in which PKC-ε cooperates with Rac, downstream of PI3K, to promote cell migration. Rac activation would promote the initial formation of lamellae whereas PKC-ε would participate in their maintenance and stability.

The data obtained in this study provide evidence for the contribution of PKC-ε to specific aspects of cell migration, i.e., the organization and stabilization of lamellae and the retraction of cell

processes. Although we excluded the involvement of PKC-ζ in cell migration, it is possible that other PKC family isoforms cooperate with PKC-ε to promote migration. In support of this, PKC-theta has been implicated in promoting vascular endothelial cell migration by a mechanism involving the regulation of actin stress fiber and filipodial formation [49]. In addition, PKC-delta may also be involved in regulating carcinoma migration [50]. The involvement of specific PKC-isoforms in migration could also be cell-type dependent. For example, activation of PKC-alpha promotes the disassembly of hemidesmosomes, very stable adhesive structures that anchor keratinocytes and epithelial cells to the underlying basement membrane (Rabinovitz, in press). Disruption of these stable adhesions facilitates epithelial cell migration. PKC-alpha has also been implicated in the loss of cell-cell adhesion of intestinal epithelial cells that preceeds the induction of migration [51]. These PKC-alpha-mediated functions may be observed only in cells that form hemidesmosomes and that maintain cell-cell contacts and therefore may not be apparent in non-epithelial cells or poorly differentiated carcinoma cells, such as the cells used in the present study. However, a careful genetic analysis of the function of all of the PKC-isoforms is needed before a complete understanding of the contribution of the PKC family to carcinoma motility and invasion is established.

## Conclusions

We have used a genetic approach to investigate the involvement of specific PKC isoforms that are known to be regulated by PI3K in carcinoma migration and invasion. We provide evidence that PKC-ε, but not PKC-ζ, is essential for migration and invasion. PKC-ε activity is required for the organization and stabilization of actin rich cell protrusions, termed lamellae, which are essential for cell migration. A role for PKC ε in retraction of cell processes is also indicated. These findings add to our current understanding of how the PKC family modifies the actin cytoskeleton in response to extracellular stimuli that promote cell migration.

## Materials and Methods

### Cells

Clone A cells were originally isolated from a human poorly differentiated colon adenocarcinoma and were obtained from D. Dexter [52]. Clone A cells were grown in RPMI supplemented with 25mM Hepes (RPMI-H), 10% fetal calf serum, 1% L-Glutamine, and 1% penicillin-streptomycin. The MDA-MB-435 breast carcinoma cell line was obtained from the Lombardi Breast Cancer Depository at Georgetown University. The transfection of the MDA-MB-435 cell line with the $\beta$4 integrin subunit has been described previously [15]. The MDA-MB-435/$\beta$4 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum (Gibco), 1% L-Glutamine, 1% penicillin-streptomycin (Gibco), and 400ug/ml G418.

### cDNA constructs

The wild-type and kinase inactive PKC-$\zeta$ plasmid constructs have been described previously [45, 53]. The human PKC-$\epsilon$ cDNA was cloned into the mammalian expression vector pcDNA3 (Invitrogen), with the addition of the FLAG epitope (DYKDDDDK) at the N-terminus. The PKC-$\epsilon$ kinase inactive mutant (KI; Lys437Trp) and activation loop T566A (Thr566Ala) mutant were generated by PCR based site-directed mutagenesis using Quickchange (Stratagene). All vector sequences were confirmed by DNA sequencing.

### Transient Transfections

Cells were passaged one day prior to transfection and plated at 80% confluency. Cells were co-transfected with either 1ug pCS2-(n)$\beta$-Gal or Green Fluorescent Protein (GFP;Clontech) and the cDNAs specified in the Figure Legends using Lipofectamine (Gibco) according to manufacturers instructions. Cells were harvested 24 hrs after transfection for experiments. To confirm the expression of the transfected proteins, cell extracts from the transfected cells were immunoprecipitated with a FLAG-specific mAb (M2;Sigma). The immunoprecipitates were

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resolved by electrophoresis on SDS-polyacrylamide gels (8%) and transferred to nitrocellulose. The tagged proteins were detected by immunoblotting with the PKC-ε (Santa Cruz) and –ζ (Santa Cruz) specific antibodies.

## Kinase Assays

Protein kinase activity of the PKC-ε and PKC-ζ proteins was assayed in transiently transfected Clone A and MDA-MB-435/β4 cells. Cells were transfected as described above and then maintained in growth medium containing 10% FBS. After washing with ice-cold 1X PBS, the cell monolayers were lysed in a 1% NP-40 lysis buffer as described previously [45]. After clearing the lysate at 14,000rpm, 5% of the total cell lysate was boiled in SDS sample buffer and the rest of the lysate was snap-frozen in liquid $N_2$ and stored at -70°C. Relative expression levels of the PKC-ε and PKC-ζ proteins were detected by SDS PAGE followed by immunoblotting with PKC-ε- (Transduction labs) and PKC-ζ- (Santa Cruz) specific antibodies. The immunoblot was exposed to a Biorad Molecular Imager chemiluminescence screen and the relative amounts of each of the PKC proteins were quantitated. Cell lysates containing equivalent amounts of PKC protein were immunoprecipitated with the FLAG-specific antibody (M2, Sigma) and a 50/50 mix of Protein A/G (Santa Cruz) for 3 hours at 4°C. The immunoprecipitates were washed stringently and subjected to an *in vitro* kinase assay using Histone H2B as a substrate (Boehringer Mannheim) as described [45]. Incorporation of 32P-[ATP] was detected by autoradiography.

## Cell migration assays

Haptotactic migration assays were performed using 6.5mm Transwell chambers (8um pore size; CoStar) as described previously [54]. Briefly, RPMI-H containing 15 ug/ml laminin (0.6ml) was added to the bottom wells of the Transwell chambers and the filters were coated for approximately 45 minutes at 37°C. Cells were resuspended in the RPMI-H containing 0.1% BSA at a concentration of $10^6$/ml and 100ul of the cell suspension were added to the top well of the Transwell chambers. After a 5 hr incubation, the cells that had not migrated were removed from

the upper face of the filters using cotton swabs. The cells that had migrated to the lower surface of the filters were fixed in 4% formaldehyde for 30 minutes and then stained with PBS containing 1mg/ml Bluo-gal (Boehringer Mannheim), 2mM $MgCl_2$, 5mM Potassium Ferrocyanide, and 5mM Potassium Ferricyanide. Migration was quantitated by counting using brightfield optics with a Nikon Diaphot microscope. All of the β-galactosidase positive cells were counted for each well.

## Invasion Assays

Matrigel invasion assays were performed as described previously [15] using 6.5mm Transwell chambers (8um pore size; Costar). Matrigel, purified from the EHS tumor, was diluted in cold distilled water, added to the upper wells of the Transwell chambers (2ug/well), and dried in a sterile hood. The Matrigel was reconstituted with medium for an hour at 37°C before the addition of cells. Cells were resuspended in serum-free medium containing 0.1% BSA at a concentration of $1-2 \times 10^6$ cells/ml and 100ul of the cell suspension was added to each well. Conditioned NIH-3T3 medium (600ul) was added to the bottom wells of the chambers. After 5 hrs, the cells that had not invaded were removed from the upper face of the filters using cotton swabs. The cells that had invaded to the lower surface of the filters were fixed, stained, and counted as described above for the migration assays.

## Indirect Immunofluorescence Microscopy

Clone A cells that had been transiently transfected with the PKC constructs were trypsinized, washed two times with RPMI containing 0.1% BSA, and plated onto coverslips that had been coated overnight with 20ug of EHS laminin. After a 45 minute incubation, the medium was removed and the cells were fixed by the addition of a 10mM Pipes buffer, pH 6.8, containing 4% paraformaldehyde, 100mM KCl, 2mM EGTA, 2mM $MgCl_2$, and 7% sucrose. After washing three times with PBS, the cells were permeabilized for 2 minutes by the addition of the same buffer lacking the paraformaldehyde and containing 0.05% Triton-X-100. The coverslips were incubated for 30 minutes in blocking buffer (PBS containing 3% BSA and 1% goat serum) and then for 30

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minutes in blocking buffer containing a 1:200 dilution of anti-FLAG mAb. After three 10 minute washes in PBS, the coverslips were incubated for an additional 30 minutes in blocking buffer containing a 1:200 dilution of Cy2-conjugated anti-mouse IgG (Jackson Laboratories). The cells were visualized by confocal microscopy (Bio-Rad).

## Video Microscopy

Cells that had been transiently co-transfected with GFP and the PKC cDNA constructs were plated on laminin coated coverslips in 60mm culture dishes. After 15 minutes at 37°C in a humidified atmosphere, the dishes were sealed with parafilm and then placed on a microscope stage heated to 37°C. An inverted microscope (model Diaphot 300; Nikon, Inc., Melville, NY) with phase contrast optics was used for image analysis. This microscope was connected to a CCD camera (Dage-MTI, Michigan City, IN), a framegrabber (Scion, Frederick, MD), and a G3 Power Macintosh computer to capture the images. GFP positive cells were identified and then the images were collected at 1 minute intervals for 30-60 minutes.

## Acknowledgements

## Figure Legends

Figure 1: **Analysis of PKC involvement in migration of Clone A cells by transient transfection.** A) Clone A cells were assayed for their ability to migrate toward a laminin-1 gradient after transient transfection with PKC cDNAs. Cells were transiently transfected with 1ug pCS2-(n)β-gal and 4ug of either the vector alone, WT-ε, KI-ε, WT-ζ, or KI-ζ. Transfected cells $(10^5)$ were added to the upper wells of Transwell chambers and medium containing 15ug/ml of laminin-1 was added to the bottom wells. After 5 hrs at 37°C, the cells that had not migrated were removed and the cells that had migrated to the lower surface of the filters were fixed, stained, and quantitated as described in Materials and methods. Expression of the transfected proteins was detected by immunoprecipitating with a FLAG-specific mAb and then immunoblotting with PKC-isoform-specific antibodies (lower panel). The data shown are the mean values (±SEM) of six (ε) or four (ζ) experiments done in duplicate. B) *In vitro* kinase assays were performed to assess the relative activity of the PKC proteins in the Clone A cells. PKC activity was assayed on a FLAG immunoprecipitate using Histone H2B as a substrate. WT, wild type; KI, kinase inactive.

Figure 2: **Morphological analysis of Clone A cells transfected with PKC-ε and -ζ.** Clone A cells that had been transiently transfected with the indicated PKC cDNAs were allowed to adhere to laminin-1 for 45 minutes. The cells were fixed and stained for the expression of the transfected PKC proteins using a FLAG-specific Ab followed by a Cy2-conjugated anti-mouse antibody as described in Materials and methods. The cells were visualized by confocal microscopy (Bio-Rad). Two representative images from each transfection are shown. A and B) Cells transfected with WT-ε display broad, polarized lamellae with extensive membrane ruffles. PKC-ε is concentrated in the membrane ruffles (arrows). C and D) Cells transfected with KI-ε lack polarized lamellae and display numerous filipodial-like structures (arrowhead). KI-ε is concentrated at the membrane edge (arrows). E and F) Cells transfected with WT-ζ display broad, polarized lamellae but PKC-ζ is not concentrated in the membrane ruffles (arrow). G and H) Cells transfected with KI-ζ display a similar morphology to the WT-ζ expressing cells. Bars, 10uM.

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Figure 3: **Videomicroscopic analysis of Clone A cells expressing WT- and KI-ε on laminin-1.** Clone A cells were co-transfected with a vector containing GFP and a vector containing either WT- or KI-ε. The transfected cells were plated on a laminin-1 substrate and GFP positive cells were analysed by time-lapse videomicroscopy. Images were obtained using a Nikon Diaphot 300 inverted microscope with phase contrast optics. This microscope was connected to a CCD camera (Dage-MTI), a frame-grabber (Scion) and a 7600 Power Macintosh computer to capture the images. A) Clone A cells expressing WT-ε form polarized, leading edge lamellae with membrane ruffles (white arrow) and these cells migrate in the direction of lamellae formation. B) Clone A cells expressing KI-ε form small, non-polarized lamellae that are less stable than the lamellae formed by the WT-expressing Clone A cells (arrowhead). The cells expressing KI-ε also exhibit defects in the retraction of cell processes (arrow).

Figure 4: **Analysis of PKC involvement in invasion of MDA-MB-435/β4 cells by transient transfection.** A) MDA-MB-435/β4 cells were assayed for their ability to invade Matrigel after transient transfection with PKC cDNAs. Cells were transiently transfected with 1ug pCS2-(n)β-gal and 4ug of either the vector alone, WT-ε, KI-ε, T566A-ε, WT-ζ, or KI- ζ. Transfected cells ($10^5$) were added to the upper wells of Matrigel-coated Transwell chambers and conditioned 3T3-media was added to the bottom wells. After 5 hrs at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed, stained, and quantitated as described in Materials and methods. Expression of the transfected proteins was detected by immunoprecipitating with a FLAG-specific mAb and then immunoblotting with PKC-isoform-specific antibodies (lower panel). The data shown are the mean values (±SEM) of six (WT-ε and KI-ε) or four (T566A-ε, WT-ζ and KI-ζ) experiments done in triplicate. B) In vitro kinase assays were performed to assess the relative activity of the PKC proteins in the MDA-MB-435/β4 cells. PKC activity was assayed on a FLAG immunoprecipitate using Histone H2B as a substrate. WT, wild type; KI, kinase inactive.

Figure 5: **Analysis of endogenous PKC-ε expression.** Total cell extracts from Clone A and MDA-MB-435/β4 cells containing equivalent amounts of protein were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with PKC-ε specific antibodies. A low level of PKC-ε expression was detected in the Clone A cells in comparison to the level of PKC-ε protein detected in the MDA-MB-435/β4 cells. In addition, a more slowly migrating, phosphorylated form of PKC-ε was detected in the MDA-MB-435/β4 cells and not in the Clone A cells. Cl A, Clone A cells; 435/β4, MDA-MB-435/β4 cells.

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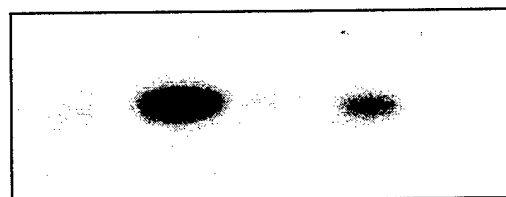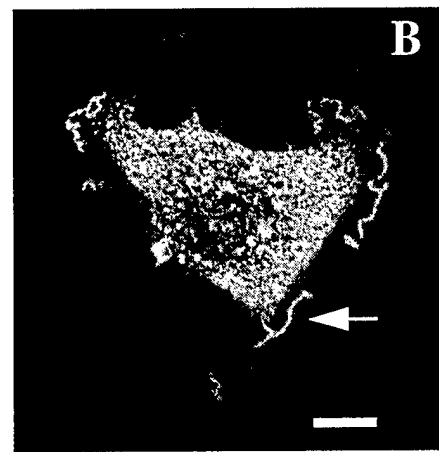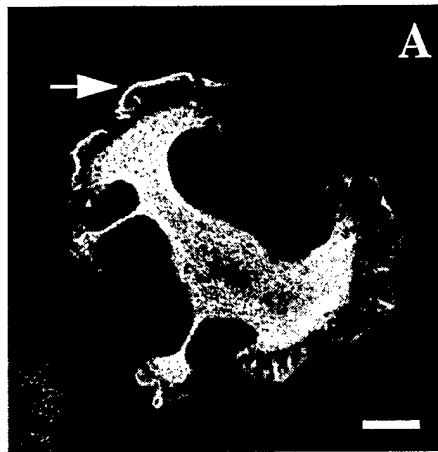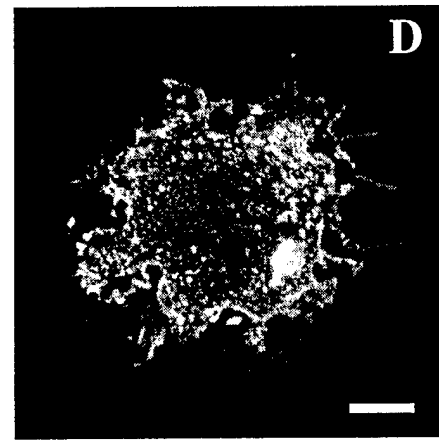
# A.



# B.
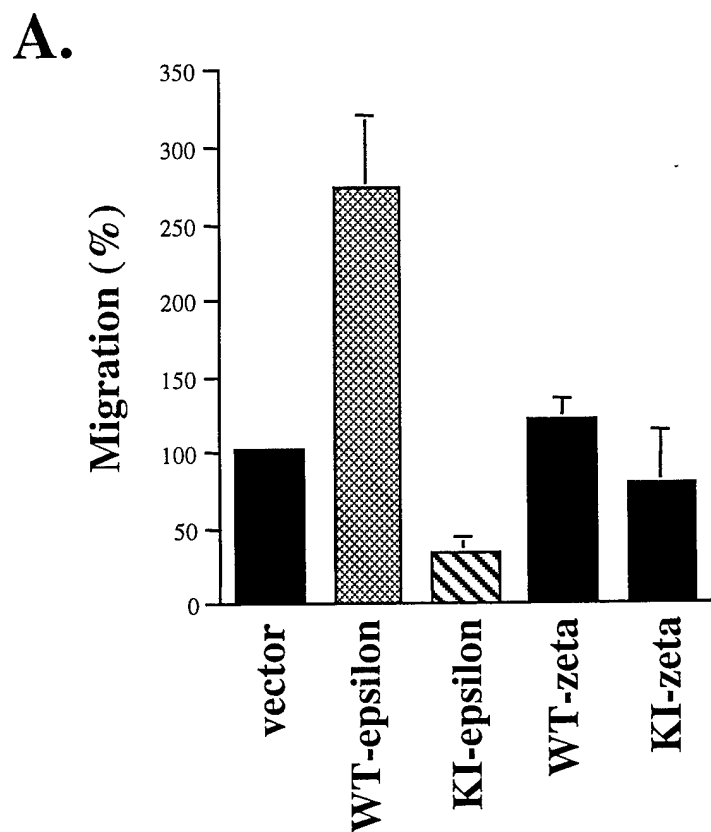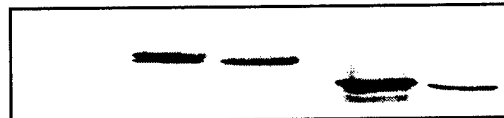


Figure 1

WT
Epsilon

KI
Epsilon
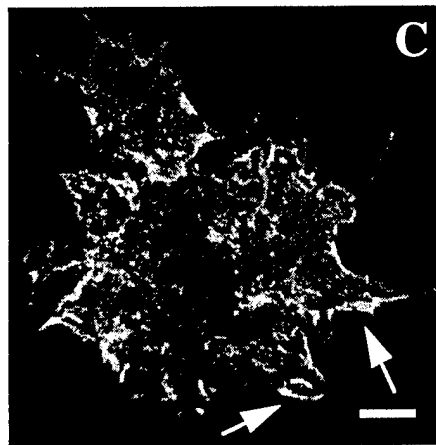
WT
Zeta

KI
Zeta

Figure 2

Figure 3

# A.



**FLAG**

# B.

**Histone H2B**

Figure 4

Cl A    435/β4

PKCε →
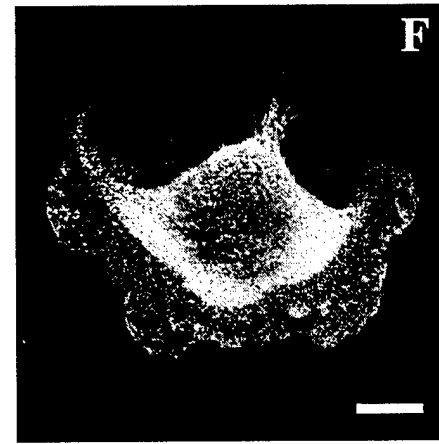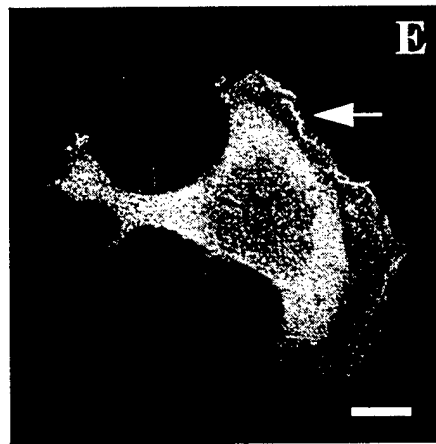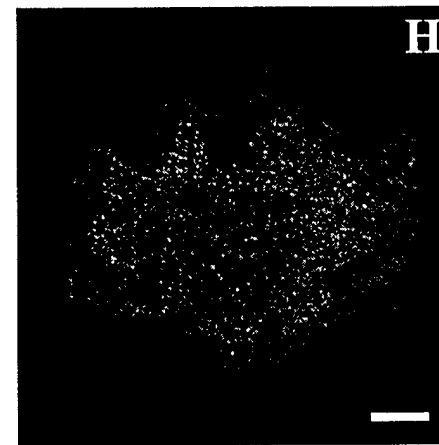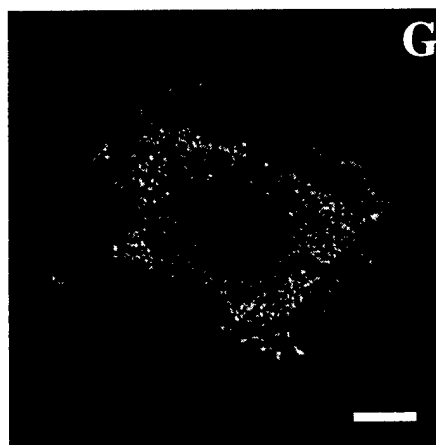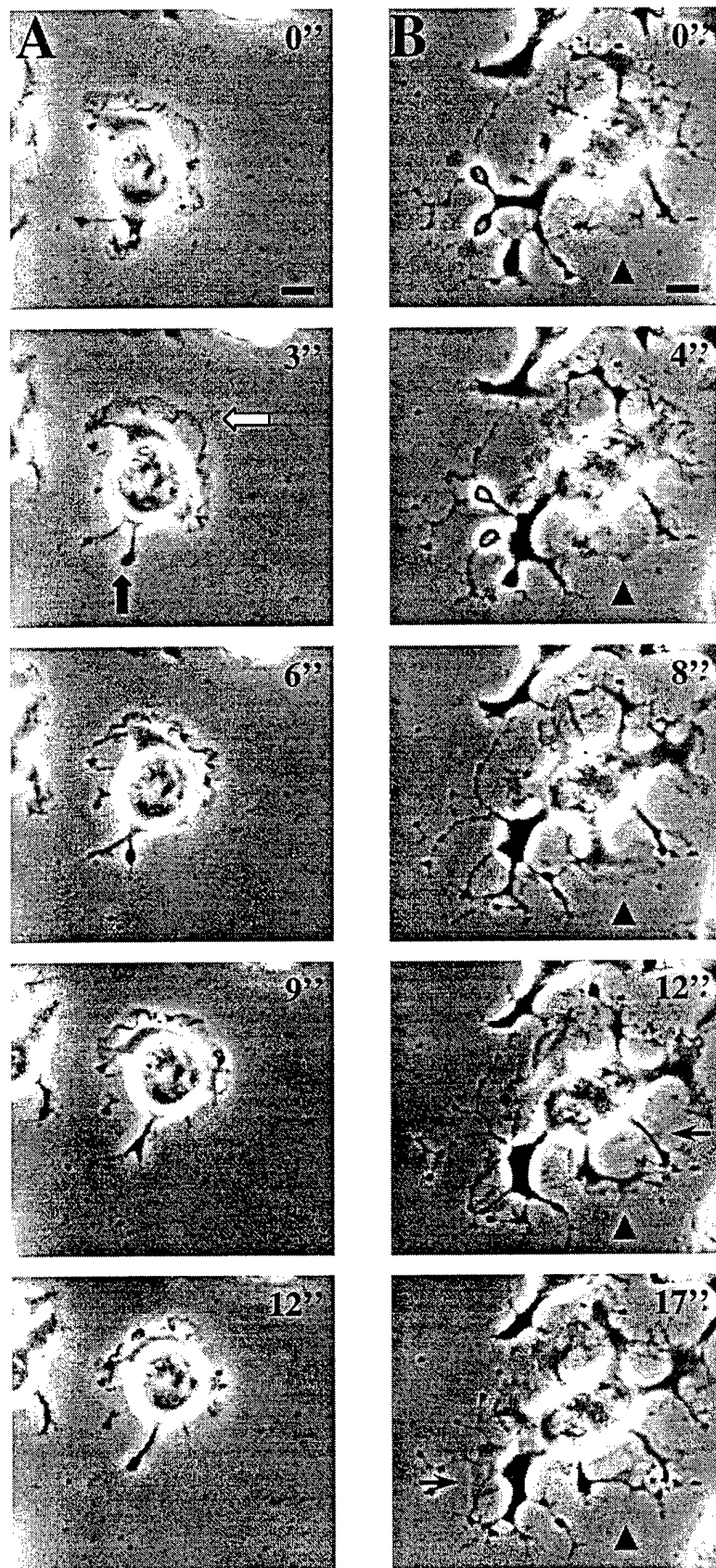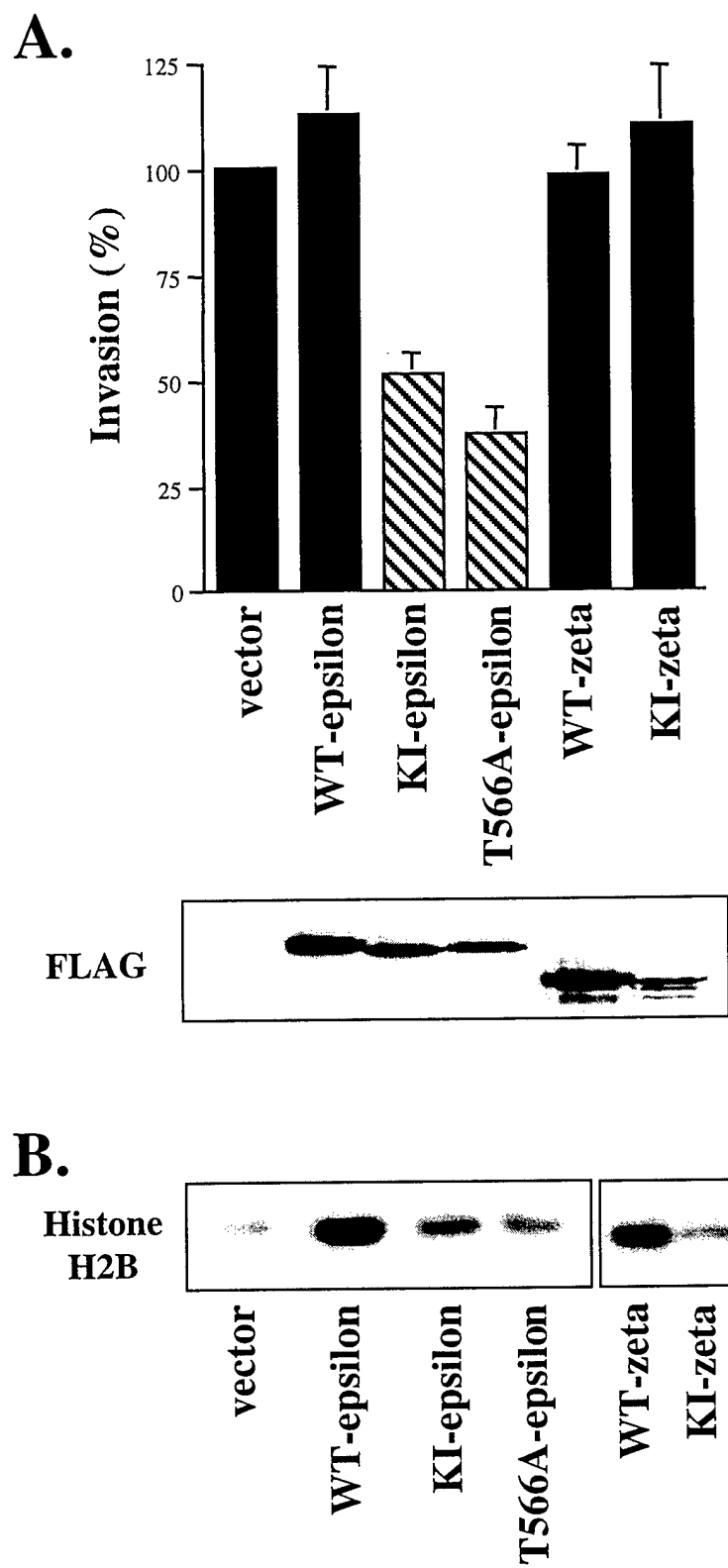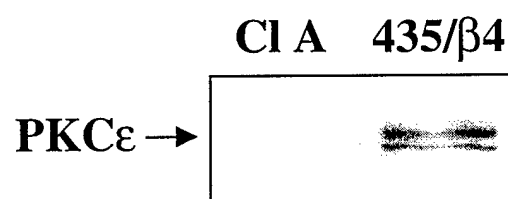
Figure 5

# Integrin Function in Breast Carcinoma Progression

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Running Head:  Integrin function in breast carcinoma progression.

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## Abstract

The differentiation and function of mammary epithelial cells is dependent upon the combined action of growth factor/hormone receptors and integrin receptors, which act in concert to control the signals required for normal cell function. It is now becoming clear that integrin receptors also contribute to carcinoma cell behavior and that alterations in expression and function during transformation have a large impact on breast carcinoma progression. The focus of this review is to discuss integrin-dependent functions that can be manipulated as targets for the therapeutic intervention of breast cancer. A combination of correlative and mechanistic studies have contributed to the identification of specific integrin receptors, namely $\alpha2\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$, that have been implicated in breast carcinoma progression. Although this field is still emerging and much remains to be learned, potential integrin-dependent signaling targets have been identified.

**Key Words:** Integrin Receptors, Invasion, Breast carcinoma, Phosphoinositide 3-OH Kinase, Extracellular Matrix

## Abbreviations

IGF-1, insulin-like growth factor-1; PI3K, phosphoinositide 3-OH kinase; [cAMP]i, intracellular cAMP concentration; EGFR, epidermal growth factor receptor.

The integrins are a family of cell-cell and cell-extracellular matrix receptors that play important roles in many cellular functions (1). These αβ heterodimers interact with their extracellular ligands as well as with the intracellular cytoskeleton to relay information in both directions across the plasma membrane (1). In addition to their adhesive functions, integrins can activate intracellular signaling pathways that regulate growth, differentiation, cell motility, and gene expression (2, 3). Many of the signaling pathways that have been characterized for growth factor receptors can be stimulated by integrin receptor engagement. In fact, these two receptor systems may act in concert to elicit the specific signals that are required for proper cell function (4). Therefore, it is not surprising that alterations in integrin expression and function during transformation are likely to have a large impact on carcinoma progression (5, 6). The goal of this review is to identify specific integrins and integrin-regulated signaling pathways that can be manipulated as targets for therapeutic intervention of breast cancer. As will be evident, this is an emerging area and much remains to be learned about integrin function in breast carcinoma, as well as other carcinomas. However, the available data highlight the potential importance of integrins in breast cancer and, hopefully, the themes that emerge from this review will provide a framework upon which future investigations can continue to build.

**Integrin expression in the normal breast.**

An understanding of integrin expression and function in the normal breast provides a foundation to study these parameters in breast cancer progression. It should be noted at the outset that there are several caveats to using integrin expression data to develop models of integrin contribution to cellular function. Most importantly, the expression of a particular integrin does not confirm that this receptor is functional. There are numerous examples of inactive integrins that require exogenous stimuli to activate binding to their ligands (1). Furthermore, we do not at present know what level of expression is sufficient for an integrin receptor to alter cellular function. Nonetheless, identifying the repertoire of

integrins that are expressed in a given tissue is a starting point from which to develop more mechanistic studies. A number of investigators have examined the expression of integrin subunits in the normal mammary gland using immunohistochemistry and a general concensus has emerged (Table 1).

The mammary epithelium is composed of two major cell types, myoepithelial and luminal epithelial cells. These cells have unique functions that are reflected in their expression of integrin receptors. Myoepithelial cells of the breast express the $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha v$, $\alpha 6$, $\beta 1$, and $\beta 4$ subunits (7-10). These integrin subunits are polarized primarily at the basal surface where interactions with their extracellular matrix ligands occur. Luminal epithelial cells express a similar panel of integrin subunits, albeit at lower levels than observed for myoepithelial cells (8, 9). Interestingly, the $\alpha 1\beta 1$ integrin, which is expressed primarily in smooth muscle cells, is expressed only in myoepithelial cells of the breast and reflects their contractile nature (11). The $\alpha 1$ subunit is expressed at the onset of myoepithelial cell differentiation and its expression is maintained throughout development and in the adult. Therefore, the $\alpha 1\beta 1$ integrin may contribute to the maintenance of the smooth muscle differentiation of this specialized epithelial layer (11). The fact that breast carcinomas rarely arise from the myoepithelium and the observation that $\alpha 1\beta 1$ is not expressed in most breast carcinomas discount a role for this integrin in the pathogenesis of this breast cancer.

Myoepithelial cells also express the $\alpha 6\beta 4$ integrin, a receptor for members of the laminin family of extracellular matrix proteins, at much higher levels than luminal epithelial cells (8, 9). The $\alpha 6\beta 4$ integrin is localized on the basal surface of myopeithelial cells within discreet, adhesive structures, termed hemidesmosomes, which are responsible for the stable adhesion of epithelial monolayers to the underlying basement membrane (12-16). In these structures, the $\alpha 6\beta 4$ integrin interacts with the cytokeratin cytoskeleton (15, 16). Although hemidesmosomes were originally characterized in stratified epithelia, both myoepithelial and luminal mammary epithelial cells form hemidesmosomes at sights of

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attachment to the basement membrane (17). In the luminal cell layer, a higher level of $\beta 4$ expression is found in the aveolar luminal cells than in the ductal luminal cells, a pattern that reflects the more frequent contact of these cells with the basement membrane (11).

**Integrin function in the normal breast.**

The differentiation and function of the mammary gland is dependent upon the concerted action of both soluble factors, such as hormones and growth factors, and insoluble factors, such as extracellular matrix proteins (18, 19). A classic example is the requirement *in vitro* of certain normal mammary cells to be grown in the presence of laminin-1 and lactogenic hormones to differentiate and express milk protein genes (20). The dependence of these cells on laminin is mediated through $\beta 1$ integrin receptors (21). Detailed studes have been performed to examine this cooperative effect. One mechanism by which matrix interactions regulate milk protein synthesis is indirect and it involves the transcriptional control of inhibitors of milk protein genes. The expression of TGF-$\beta 1$ and TGF-$\alpha$, which inhibit the transcription of $\beta$-casein and whey acidic protein, is strongly inhibited by integrin-dependent interactions with the extracellular matrix (22, 23). Another mechanism of cooperativity is shown in prolactin stimulation of transcription of $\beta$-lactoglobulin mRNA through activation of the transcription factor, Stat5. In the absence of laminin-1, Stat5 binding activity for the $\beta$-lactoglobulin gene is not induced (24). In fact, prolactin does not induce phosphorylation of its receptor or the associated signaling kinase, Jak2, unless the cells interact with laminin-1, an effect that may be controlled through integrin regulation of a protein tyrosine phosphatase(s) (25). These examples of cooperativity between integrins and growth factors/hormones emphasize the contribution of integrin receptors to the normal biology of mammary epithelial cells.

It is well documented that disregulation of growth factor receptor signaling pathways, either through changes in expression levels or oncogenic activation, contributes to breast cancer progression (26). Given the complex nature of the relationship between

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these two receptor systems, it is not surprising, therefore, that alterations in either the expression or function of integrin receptors also disturb the balance required for normal differentiation and promote tumor progression.

**Integrin expression and function in breast carcinoma.**

The contributions of integrin receptors to breast carcinoma progression have been investigated in numerous immunohistochemical studies, as well as in more recent mechanistic studies (Table 1). When reviewing the data on integrin expression in breast cancer it is important to keep in mind that breast cancer arises primarily from luminal epithelial cells and very rarely from cells of the myoepithelial lineage (27). In the normal breast, intense staining for many of the integrin subunits is seen concentrated at the basement membrane in the myoepithelial layer, as mentioned above (28). In invasive carcinomas, this cell layer is most often absent and the expression of integrin subunits on the surface of carcinoma cells is diffuse. This staining pattern has led to the erroneous assumption that there is an overall decrease, or absence, of integrin expression in breast carcinoma. Although the expression of some integrin subunits is decreased, it is clear from many *in vitro* and *in vivo* studies that integrin receptors are expressed in breast adenocarcinomas and that they contribute significantly to the pathobiology of breast cancer. The integrin receptors that have been most clearly implicated in breast cancer progression are $\alpha2\beta1$ and the $\alpha6$ integrins ($\alpha6\beta1$ and $\alpha6\beta4$).

**The $\alpha2\beta1$ integrin.**

The $\alpha2\beta1$ integrin is a dual-specificity receptor that recognizes collagens I and IV and members of the laminin family (1). The $\alpha2$ subunit is expressed basally as well as laterally in luminal epithielial cells (8, 10). In the latter location, it may contribute to cell-cell interactions as it has been suggested to do in other epithelial cell types (29). The expression of $\alpha2\beta1$ is maintained in benign breast lesions such as fibrocystic disease or

fibroadenomas (8, 10). However, the expression level of α2β1 decreases with the differentiation status of breast adenocarcinomas. Specifically, poorly differentiated adenocarcinomas express very low, or undetectable levels of α2β1 while moderately differentiated adenocarcinomas express intermediate levels (10, 30). These correlative studies suggest that the α2β1 integrin is important for maintaining differentiation and controlling proliferation of the breast epithelium and that its loss is essential for the progression to invasive carcinoma.

The possibility that α2β1 contributes to mammary epithelial differentiation has been addressed more rigorously by studies in which the contribution of the α2β1 receptor to breast carcinoma function has been examined *in vitro*, as well as in *in vivo* model systems. Downregulation of α2 subunit expression in a well-differentiated breast carcinoma cell line using antisense oligonucleotides resulted in a transition from a contact-inhibited epithelioid morphology to a more mesenchymal, fibroblastic morphology indicative of a poorly differentiated carcinoma (31). Conversely, re-expression of α2 in a poorly differentiated, invasive breast carcinoma cell line caused the cells to regain their contact inhibition, form cell-cell contacts, and diminish their *in vitro* invasive potential (32). More importantly, the *in vivo* tumorigenicity of the α2-expressing cells was dramatically reduced (32). Taken together, these results support a role for α2β1 in regulating the differentiation of the breast epithelium and suppressing tumorigenicity and invasion. The signaling pathways that are activated by the α2β1 integrin to promote mammary epithelial differentiation have not been identified. However, the information gained from these analyses should contribute greatly to our understanding of the early stages of breast carcinoma progression.

Interestingly, the mechanism by which the α2β1 integrin modulates differentiation may be related, in part, to the up-regulation of the α6 and β4 integrin subunits. The expression of these two subunits, but not other integrin subunits, was significantly enhanced when the α2 subunit was overexpressed in poorly differentiated breast carcinoma cells (33). When either the α6 or β4 subunit was overexpressed independently of the α2

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subunit many, but not all, of the differentiated characteristics that were observed for the $\alpha 2$ transfectants were recapitulated (33). For example, the $\alpha 6$ and $\beta 4$ transfectants became contact inhibited and their growth was diminished, but they did not form highly branched, duct-like structures when grown within a collagen gel. In addition, the $\alpha 6$ and $\beta 4$ transfectants were more invasive *in vitro* than the parental cell line contrasting with decreased invasion observed for the $\alpha 2$ transfectants. This involvement of the $\alpha 6 \beta 4$ receptor in carcinoma invasion is discussed below.

**The $\alpha 6 \beta 1$ integrin.**

There are conflicting data in the literature with regard to the expression of the $\alpha 6$ subunit in breast carcinoma. As with the $\alpha 2 \beta 1$ receptor, the level and the pattern of expression of the $\alpha 6$ subunit in benign lesions such as fibrocystic disease and fibroadenomas are similar to those observed for the normal mammary gland (8). $\alpha 6$ expression is strongest in the myoepithelial cells and the staining is predominantly polarized at the basal surface in contact with the basement membrane. However, disagreements arise in the analysis of expression levels of the $\alpha 6$ subunit in adenocarcinomas of the breast. A loss or absence of $\alpha 6$ staining in poorly differentiated invasive ductal and lobular carcinomas has been described in several studies (34-36). In contrast, other reports suggest that $\alpha 6$ expression is retained in many breast adenocarcinomas and the localization of expression suggests an important role in malignancy (7, 8, 37). For example, consistent staining of the $\alpha 6$ subunit in pseudopodial-like extensions of invasive lobular carcinoma suggests a possible role for this integrin in the motile phenotype of these cells (7, 8).

One difficulty in assessing the importance of integrin receptors to breast carcinoma progression based upon expression patterns alone is that the data are not usually correlated with patient outcome or survival. However, Imhof and colleagues reported that high expression of the $\alpha 6$ subunit in women with breast cancer correlated significantly with reduced survival times (37). In their analysis of 119 patients with invasive breast

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carcinoma, all of the patients with low or absent α6 expression survived while the mortality rate of the patients with a high level of α6 expression was 19%. These correlations with survival were stronger, in fact, than those observed with estrogen receptor status. Of note, 30 out of 34 of the patients who presented with distant metastases were highly positive for α6 expression. Taken together, these analyses provide strong support for a role for α6 containing integrin receptors in the promotion of metastatic breast cancer.

It must be remembered that the α6 subunit can associate with both the β1 and β4 subunits to form the α6β1 and α6β4 receptors (1). The results from the Imhof study do not distinguish between these two combinations leaving open the question of whether one or the other, or both, are responsible for promoting the metastatic potential of breast carcinoma. Recently, results of a similar study examining the correlation of β4 expression with survival rates of breast cancer patients were reported. Patients with tumors that expressed both the β4 subunit and specific laminins, ligands for both α6β1 and α6β4, had increased mortality rates (38). This study highlights an additional caveat to using expression data alone to interpret integrin function, *i.e.*, if the appropriate extracellular matrix ligand is not present, integrin receptors may be expressed but not functional. Although the findings support the involvement of α6β4 in tumor progression, they do not negate a role for α6β1 because immunohistochemical analyses cannot distinguish relative levels of α6β1 and α6β4 on the cell surface. Fortunately, several mechanistic studies have begun to address this important question and it appears that both the α6β1 and α6β4 integrins can play important roles in breast cancer progression.

To address the contribution of the α6β1 receptor to breast cancer, a dominant-negative technique for 'knocking-out' the expression of α6β1 in MDA-MB-435 cells, a human breast carcinoma cell line that is highly metastatic in athymic mice, was developed (39). A mutant β4 cDNA that lacked most of the cytoplasmic domain was transfected into the MDA-MB-435 cell line, which does not express the β4 subunit. The truncated β4 subunit acted as a dominant negative by associating with the endogenous α6 and depleting

functional α6β1 on the cell surface. Elimination of α6β1 expression inhibited the ability of these cells to mediate specific *in vitro* functions associated with metastatic spread such as laminin-1 adhesion and migration (39). When these cells were inoculated into the mammary fat pad of nude mice, primary tumor size was significantly diminished because of a combined decrease in proliferation and increase in apoptosis (40). More importantly, the α6β1 deficient cells could not survive as secondary metastases in the lungs or liver because they underwent apoptosis (40). Taken together, these data indicate the α6β1 integrin contributes to breast carcinoma progression by promoting the growth and survival of breast carcinomas. It should be noted that the α6β1 receptor has also been implicated in the binding of breast carcimoma cells to the endothelium, a function that could also contribute to its promotion of metastasis (41).

The specific signaling pathways that are regulated by α6 to promote the growth and survival of mammary carcinoma cells could be important targets for inhibitors of metastasis. Although these pathways remain to be elucidated, a clue may be found in a recent report that described a role for the α6β1 receptor in the survival of normal mammary epithelial cells (42). These cells require both α6β1 ligation and insulin or insulin-like growth factor-1 (IGF-1) to prevent apoptosis when they are grown *in vitro*. Ligation of α6β1 does not influence the phosphorylation of the growth factor receptors but it does increase the activation of downstream signaling molecules including the insulin receptor substate-1, the lipid kinase phosphoinositide 3-OH kinase (PI3K), and the serine/threonine kinase Akt/PKB. Activation of PI3K was essential for the survival effect. This finding adds to the growing number of studies that support the contribution of PI3K and Akt/PKB to the survival of many different cells (43-45). With regard to the survival of breast carcinoma cells, the discovery that α6β1 cooperates with IGF-1 to promote survival is intriguing because the IGF-1 receptor has been implicated in the survival of many different tumor cell types, including breast (46). It is not unlikely that a pathway involved in normal cell function would be utilized by transformed cells and this possibility warrants further

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investigation.

## The α6β4 integrin

The role of the α6β4 integrin in breast carcinoma progression is a complex one and represents the best example of how difficult it is to understand the contribution of an integrin receptor to carcinoma progression based upon expression data alone. In normal epithelial cells, the α6β4 integrin participates in the formation of hemidesmosomes, very stable, adhesive structures that are not conducive to cell motility (15, 16, 47, 48). For this reason, it had long been thought that a loss of β4 expression would be necessary for the acquisition of the motile phenotype characteristic of invasive carcinomas. In fact, many of the early immunohistochemical reports supported this hypothesis because a decrease in β4 expression was detected (49). In contrast, as mentioned previously, more recent studies have demonstrated β4 expression in aggressive breast carcinomas (38), as well as many other types of carcinoma (49).

The mechanism by which the α6β4 integrin contributes to breast carcinoma progression has been addressed by *de novo* expression of the β4 subunit in cell lines that express α6β1 and not α6β4. When the β4 subunit was transfected into the MDA-MB-435 cell line, a 3-4 fold increase in the *in vitro* invasive potential of these cells was observed (50). As mentioned previously, this increase in invasion was also observed when β4 was overexpressed in a murine mammary carcinoma cell line (33). Similar increases in invasion after α6β4 expression have been reported for a colon carcinoma cell line, RKO (51). The apparent paradox in α6β4 function in normal epithelia and carcinomas has been resolved by the recent demonstration that the α6β4 receptor can interact with the actin cytoskeleton in invasive carcinoma cells and that α6β4 contributes to the formation of motility structures such as filipodia and lamellae in these cells (52). The concensus from these data is that the α6β4 receptor contributes to carcinoma progression through its ability to promote motility and invasion.

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The data implicating α6β4 in invasion support the role of this integrin as a tumor promoter. However, this conclusion is contradicted by the finding that expression of the β4 subunit in the RKO colon carcinoma cell line also resulted in an increase in cell death (53). These tumor-promoting and tumor-suppressing activities of α6β4 could conflict with one another and complicate the understanding of α6β4 function in carcinoma progression. Interestingly, an increased level of apoptosis was not observed in the MDA-MB-435/β4-transfected cells (54). Further analysis of the differences between the RKO and MDA-MB-435 cells has revealed that they differ in their p53 status: RKO cells express wild type p53 whereas MDA-MB-435 cells express mutant p53 (54, 55). After expression of a dominant negative p53 mutant in the RKO cells, the ability of α6β4 to enhance apoptosis was abolished (54). These exciting findings underscore the fact that integrin expression alone cannot be used to assess function because the regulation and signaling capabilities of these receptors are influenced by many other factors.

Based on our knowledge to date, a model for α6β4 function in breast carcinoma progression can be proposed. An initial decrease in α6β4 expression, or altered α6β4 function, may be required for the early stages of tumor development if p53 is still functional. In the absence of p53 activity, re-expression or activation of α6β4 may promote carcinoma progression by enhancing the invasive phenotype and metastasis. One intriguing study that identified a primary tumor that was α6β4 deficient and an autologous lymph node lesion that was α6β4 positive is consistent with this model (35).

Given a role for α6β4 in carcinoma invasion, it is imperative to determine the mechanism involved so that methods for intervention can be developed. Investigation of the signaling pathways involved in the α6β4-dependent promotion of invasion revealed that PI3K activity was required for invasion (50). In keeping with this requirement, α6β4 activated PI3K to a greater extent than α6β1 or other β1 integrins (50). The importance of PI3K to carcinoma invasion was confirmed by the ability of a constitutively active PI3K to promote MDA-MB-435 invasion in the absence of α6β4 expression, and conversely, a

dominant negative PI3K p85 subunit to inhibit $\alpha6\beta4$-dependent invasion (50). The involvement of PI3K in breast carcinoma invasion was also demonstrated by an independent study in which the invasive potential of T47D breast carcinoma cells was diminished by inhibitors of PI3K signaling (56). Taken together, the data implicating the activity of PI3K in both $\alpha6\beta1$ and $\alpha6\beta4$-dependent functions support a central role for this kinase and its lipid products in breast carcinoma progression and highlight the need to investigate this signaling pathway in more detail for its therapeutic potential.

In addition to PI3K itself, downstream effectors that mediate the action of PI3K could also be targets for therapy. One such molecule that has been shown to be essential for PI3K-dependent invasion is the small G-protein Rac (50). Rac belongs to the Rho small G-protein family, the members of which participate in modification of the actin cytoskeleton in response to many stimuli (57). In fibroblasts, expression of constitutively active Rac results in the formation of membrane ruffles and lamellipodia, structures that are indicative of a motile phenotype (57). In carcinoma cells, lamellae formation is also dependent upon PI3K activity and Rac is essential for this function (50). However, Rac alone is not sufficient to promote the invasion of MDA-MB-435 cells indicating that other downstream effectors of $\alpha6\beta4$ and PI3K are required. In contrast, expression of constitutively active Rac, and also Cdc42, increased the invasive potential of T47D breast carcinoma cells in the absence of other stimuli (56). Although many factors could differ between these two carcinoma cell lines, a plausible explanation for this discrepancy could be related to their level of differentiation. MDA-MB-435 cells are poorly differentiated and they do not express E-cadherin or form cell-cell interactions (58). In contrast, T47D cells are more well differentiated and maintain cell-cell adhesion through E-cadherin interactions (58). In normal epithelial cells, E-cadherin-mediated cell-cell interactions can be modified by the action of Rho family members on the actin cytoskeleton (59). Therefore, given the large amount of data correlating the loss of E-cadherin with increased cell motility (60, 61), this could be the mechanism by which Rac and Cdc42 increase the invasion of T47D cells.

Nevertheless, in both systems Rac plays an important role in the invasive phenotype and it should be considered an important candidate for the development of inhibitors of invasion.

Akt/PKB is a another downstream effector of PI3K that is a potential target for the inhibition of carcinoma progression. In the absense of Akt/PKB function, cells undergo apoptosis upon growth factor withdrawal or, in the case of epithelial cells, upon detachment from the ECM (43-45). Akt/PKB function is also required for the survival of transformed cells (43). As described above, Akt/PKB activation may play a role in the $\alpha6\beta1$-dependent survival of breast carcinoma cells and, therefore, inhibition of this PI3K effector would decrease their ability to metastasize. Of note, Akt/PKB function is not required for carcinoma invasion even though it is activated by $\alpha6\beta4$ (50). This observation suggests that the PI3K effectors that are involved in invasion are distinct from those involved in survival. Given that carcinoma progression involves both tumor cell invasion and survival, disruption of either the Rac or Akt/PKB pathways should limit the development of metastases.

The ability of $\alpha6\beta4$ to promote invasion does not depend on PI3K activation alone but involves other signaling pathways as well. The recent demonstration that $\alpha6\beta4$ stimulates the chemotactic migration and invasion of breast carcinoma cells by suppressing the intracellular cAMP concentration ($[cAMP]_i$ )through the activation of a cAMP-specific phosphodiesterase (62) provides an additional target for intervention. The decreased $[cAMP]_i$ allows the propagation of chemotactic signals that would otherwise be inhibited, or "gated", at higher $[cAMP]_i$. Although there have been reports of cAMP regulating PI3K activity and both pathways are involved in lamellae formation, the data suggest that $\alpha6\beta4$ regulates PI3K and the PDE through distinct mechanisms and that they function independently of one another (50, 62).

What is the mechanism by which $\alpha6\beta4$ activates the signaling pathways that are important for carcinoma progression? The $\beta4$ subunit has an extremely long cytoplasmic domain (1000 amino acids) compared to other integrin $\beta$ subunits (30-50 amino acids) and

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it is required for the signaling functions of α6β4 (12-14, 50). Surprisingly, very little is known about the interactions of this large cytoplasmic domain as they relate to its signaling properties and the few studies that have examined the signaling functions of α6β4 have not been definitive. For example, the β4 cytoplasmic domain is phosphorylated on tyrosine, as well as serine and threonine residues upon clustering or ligation of the α6β4 receptor (63). However, the mechanism for this phosphorylation is unknown and the specific residues that are modified have not been identified. Shc has been reported to be phosphorylated and to associate with the β4 cytoplasmic domain upon ligation but its specific binding site in β4 has not been identified (63). In addition, a direct binding motif for the p85 regulatory subunit of PI3K is not present in the β4 cytoplasmic domain, suggesting that this lipid kinase is activated through intermediate signaling molecules (64). It is clear that a more detailed and rigorous analysis of the β4 subunit is needed to understand how α6β4 signals invasion. Only then will it be possible to devise methods to interfere with the activation of its specific signaling pathways that contribute to carcinoma progression.

## Integrin cooperativity with Growth Factors

An emerging area in the study of integrin contributions to cancer is the cross-talk between these adhesion receptors and soluble growth factor and cytokine receptors (4). As mentioned previously, in the normal breast integrin receptors cooperate with hormones and growth factors to promote mammary epithelial differentiation and function (18). An increasing number of studies indicate that integrins also cooperate with soluble factors to promote carcinoma progression. From these studies, several mechanisms for cooperation have been revealed that may be useful in the future manipulation of these pathways to inhibit progression.

Integrins can cooperate with growth factor receptors to enhance their signaling capabilities (4; Figure 1A). An example of this, as mentioned previously, is the

cooperation of the α6β1 integrin with the IGF-1 receptor to activate cell survival pathways in normal mammary epithelial cells (42). Integrin receptors can also associate directly with growth factor receptors to enhance signaling. One example that is especially relevant for breast cancer is the finding that the α6β1 and α6β4 integrins can associate with the ErbB2 protein and increase its signaling functions (65). This cooperation between α6β4 and ErbB2 could also be a mechanism through which α6β4 activates its own downstream signaling pathways. ErbB2 is a member of the epidermal growth factor receptor (EGFR) family and its overexpression in breast cancer correlates with a poor prognosis (66). *In vitro*, antibodies specific for ErbB2 inhibit the growth of ErbB2 overexpressing breast carcinoma cells. The recent success of a recombinant, humanized ErbB2 antibody (Herceptin) in clinical trials confirms the relevance of the ErbB2 receptor as a therapeutic target (67). It is intriguing, therefore, to consider the possibility that methods to disrupt the interaction between α6β4 and ErbB2, and interfere with signaling functions, could also have therapeutic potential.

Another mechanism of integrin-growth factor receptor cooperation involves integrin activation by growth factors and cytokines (Figure 1B). A well studied example is the activation of the αvβ5 integrin by the insulin-like growth factor receptor (68, 69). Breast carcinoma cells that express αvβ5 adhere to but do not migrate on vitronectin *in vitro* unless they are stimulated with IGF-1 or insulin. *In vivo*, these cells form tumors in the absence of IGF-1, however, they only metastasize when stimulated with IGF-1 or insulin (68). These data suggest that the cooperation between αvβ5 and the IGF-1 receptor may regulate migration and invasion to promote the metastatic spread of tumor cells.

A final intriguing mechanism for cooperation involves cross-talk between integrin and growth factor receptors regulating the expression levels of these receptors (Figure 1C). The growth and morphology of a breast carcinoma cell line cultured within a 3-D basement membrane matrix could be altered by inhibiting the function of either the β1 integrin subunit or the EGFR resulting in a concommitant decrease in the expression of the other (70). The

reciprocal modulation of the expression of these receptors was controlled through MAPK (70). This cross talk confirms the notion that the normal differentiation of the mammary epithelium is controlled by a tightly regulated balance of signaling pathways and that disruption of this balance can contribute to breast carcinoma progression.

## Summary

The purpose of this review has been to examine the contribution of integrin receptors to breast carcinoma progression and to highlight some of the possible targets of integrin signaling pathways for therapeutic development. It is clear from this analysis that specific integrins play an important role in breast cancer and that they have the potential to be manipulated for intervention in tumor progression. Several key areas emerge that deserve further examination. The cross-talk between receptors for soluble factors (growth factors, hormones, and cytokines) and integrin receptors is an exciting area that has just begun to be appreciated. Most likely, more examples of cooperativity will emerge and the challenge will be to decipher the mechanisms involved as well as the specific contributions of each to cancer progression. With regard to integrin-regulated signaling pathways, there is solid evidence to support a pivotal role for PI3K in breast carcinoma progression. The activation of this lipid kinase is required for two essential functions of progression, invasion and survival. Understanding how specific integrin receptors activate this signaling pathway, either independently or in cooperation with growth factors, will be an important focus in future studies. Finally, more detailed and mechanistic analyses of the contributions of integrin receptors to breast carcinoma progression are needed. Although strong correlations between integrins and breast cancer have been established, more work remains to unravel the specific mechanisms that are responsible for the effects of these integrins on tumor growth, progression and metastasis.

## Acknowledgements

**Table I:** Summary of integrin expression and function.

| Integrin Receptor | Myoepithelial | Luminal Epithelial | Invasive Carcinoma | Proposed Function in Carcinoma |
|---|---|---|---|---|
| α1β1 | ++ | - | - | Not expressed |
| α2β1 | ++ | + | +/- | Regulates epithelial differentiation |
| α3β1 | ++ | + | +/- | ? |
| α5β1 | + | + | +/- | ? |
| α6β1 | +++ | + | +/++ | Promotes growth and survival |
| αvβ? | + | +/- | + | ? (The association of αv with β1, β3, β5, or β6 has not been clearly established) |
| α6β4 | +++ | + | +/++ | Promotes invasion |

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## Figure Legends

Figure 1: Schematic representation of cooperativity between growth factor and integrin receptors. A). Growth factor receptor and integrin-dependent signals are amplified when both receptors are engaged. B). Integrin function is activated in response to growth factor receptor signaling. C). Cross-talk between growth factor and integrin receptors can regulate the expression levels of each receptor.